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DNA BARCODE OF WILD *Averrhoa* AND INTERSPECIFIC RELATIONSHIP OF THE GENUS *Averrhoa* INFERRED FROM NUCLEAR INTERNAL TRANSCRIBED SPACER AND *trnL-F* REGIONS

Barkoding DNA belimbing liar dan hubungan interspesifik marga Averrhoa berdasarkan daerah inti Internal Transcribed Spacer dan trnL-F

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Abstrak

Dua jenis belimbing liar (*Averrhoa dolichocarpa* Rugayah & Sunarti dan *Averrhoa leucopetala* Rugayah & Sunarti) telah dideskripsikan sebagai jenis baru pada tahun 2008. Penelitian lebih lanjut tentang pembungaan dan fenologi serta analisis fitokimia telah dilakukan terhadap kedua jenis tersebut. Namun demikian, belum ada data barkoding DNA yang tersedia untuk kedua jenis belimbing liar tersebut. Ketersediaan data barkoding DNA yang dapat diakses secara terbuka penting untuk tujuan identifikasi dan memahami hubungan interspesifik marga *Averrhoa*. Dalam penelitian ini, kami mengurutkan daerah inti *Internal Transcribed Spacer* (ITS) dan *trnL-F*. Kedua daerah ini dapat digunakan untuk membedakan kedua jenis belimbing liar dan membedakannya dari *A. carambola* dan *A. bilimbi*. Namun, kedua daerah penanda ini tidak bisa digunakan untuk membedakan *A. carambola* dari *A. bilimbi.* Hasil analisis filogenetik menunjukkan bahwa *Averrhoa* merupakan kelompok taksa yang monofiletik. Marka *ITS* dan *trnL-F* tidak dapat digunakan sebagai barkoding DNA untuk marga *Averrhoa*.

Abstract

Two species of wild starfruit (*Averrhoa dolichocarpa* Rugayah & Sunarti and *Averrhoa leucopetala* Rugayah & Sunarti) were described as new species in 2008. Further research has been carried out on those species related to their flowering, phenology, and phytochemical analysis . However, there has been no DNA barcode available for these wild starfruits. DNA barcode is important for sharing information for identification purposes and understanding the interspecific relationships of *Averrhoa*. This study sequenced the nuclear Internal Transcribed Spacer (*ITS*) and the plastid *trnL-F* regions. These two regions differentiated the two species of wild *Averrhoa* from one another, as well as from *A. carambola* and *A. bilimbi*. Nevertheless, the markers were unable to distinguish *A. bilimbi* from *A. carambola*. The results of the phylogenetic study showed that *Averrhoa* is a distinct monophyletic group. The *trnL-F* and *ITS* markers could not be used as DNA barcodes for the genus *Averrhoa*.

INTRODUCTION

Averrhoa L. is a genus in the Oxalidaceae family known for its edible starfruit, *Averrhoa carambola* L. It was first described by Linnaeus based on three woody species known since ancient times in the old continent and illustrated by Rumphius and Rheedius. The genus consists of four species, namely *A. bilimbi* L., *A. carambola* L., *A. dolichocarpa* Rugayah & Sunarti, and *A. leucopetala* Rugayah & Sunarti. A species previously known as *A. acida* L. was eventually revised as a synonym of *Phyllanthus acidus* (L.) Skeels. It was erected in its own family, Averrhoaceae by Hutchinson (1959), but this was not widely accepted by other researchers. The genus was reintegrated into Oxalidaceae by Veldkamp (1967, 1971).

The genus *Averrhoa* is identified from its indehiscent fleshy fruit and imparipinnate leaves (Veldkamp 1971). *Averrhoa* is thought to originate from the Malesia region, but it has been widely cultivated in the pantropic region. The four species of *Averrhoa* are

found in Indonesia, with *A. carambola* and *A. bilimbi* have been consumed as fruits, spices, and herbs. The other two species, *A. dolichocarpa* and *A. leucopetala* were described in 2008, and known as wild starfruits (Rugayah & Sunarti 2008).

A. dolichocarpa was found in Papua, while *A. leucopetala* was found in Gorontalo. Both species were collected from where they were found and planted in the Bogor Botanic Gardens. Wild starfruit species can be distinguished from their cultivated relative species by characters of the flowers, inflorescences, leaves and fruits (Rugayah & Sunarti 2008). Many studies have been done to uncover the characteristics of these two wild species. Leaf anatomical observations indicated that the four *Averrhoa* species showed variations in the thickness of their lamina and epidermal cells (Sunarti *et al.* 2008). Genetic diversity and genetic relatedness studies confirmed that the two wild *Averrhoa* species are genetically distant from *A. carambola* and *A. bilimbi* (Yulita 2011). Pollen observations showed that the pollen shape of *A. leucopetala* and *A. dolichocarpa* is tricolpates with a smooth surface on *A. leucopetala* and a rough surface on *A. dolichocarpa* (Kapsah *et al.* 2016). Mangunah *et al.* (2013) observed the phenology of *A. dolichocarpa* and *A. leucopetala.* The inflorescence initiation time of *A. dolichocarpa* was 8–14 days, single flower bud phase was 11–15 days, large bud phase was 1 day, anthesis phase was 3 days, and fruit development was 40–45 days. Whereas, those of *A. leucopetala* were 30–34 days, 12–15 days, 1 day, 5 days, and 40–42 days, respectively.

To date, no DNA barcode sequence data are available. DNA barcode is important for establishing a shared community resource of DNA sequences that can be used for organismal identification. In this study, we sequenced the nuclear Internal Transcribed Spacer (ITS) and the plastid *trnL-F* (*trnL* intron and *trnL–trnF* (GAA) Intergenic Spacer) of *A. leucopetala* and *A. dolichocarpa.* We also analyzed the interspecific relationship of the *Averrhoa* genus. The *trnL* intron and *trnL-trnF* (GAA) Intergenic Spacer is a non-coding region expected to mutate faster than the coding region and thus provides better resolution for low-level systematics. Meanwhile, the ITS region of the nuclear ribosomal DNA repeating unit evolves rapidly and may vary between species within the genus (Schoch *et al.* 2012). The ITS marker has been widely used as a universal barcode marker for plants. A better understanding of the characteristics of the wild species of *Averrhoa* can be useful for the improvement of neglected and underutilized plants, *A. carambola* and *A. bilimbi*, given that wild relatives are a source of genetic materials.

MATERIALS AND METHODS

Plant materials

Fresh leaf samples were collected from living plant collections of *A. dolichocarpa* and *A. leucopetala* at Bogor Botanic Gardens. The leaves were kept in tea bags and preserved using silica gel. Leaves of *Averrhoa* spp. were also collected from herbarium specimens using loose leaves kept in the envelopes. A total of 26 DNA sequences were obtained from the 15 samples in this study (Table 1). In addition, we also obtained more DNA sequences of *A. bilimbi, A. carambola,* as well as sequences of *Dapania pentandara* and *Sarcotheca monophylla* for the outgroup from NCBI GenBank (Table 1).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from leaf samples using the Plant Genomic DNA Mini Kit (GeneAid) following the product protocol. We amplified the ITS region using ITS5 and ITS4 primers for fresh leaf samples and using two pairs of primers (ITS1-ITS2 and ITSS2F-ITS4) for herbarium samples (White *et al.* 1990; Chen *et al.* 2010). The *trnL-F* region was amplified using c, d, e, and f primers (Taberlet *et al.* 1991). Polymerase chain reactions (PCR) were carried out on TaKaRa Dice (TP600) PCR Thermal Cycler (TAKARA) in a 12.5 µl volume containing ~ 20 ng/µl DNA, 2x PCR buffers, 0.4 mM dNTPs, 0.3 µM forward and reverse primers, and 1.0 U / 50 µL KOD FX (TOYOBO). The PCR condition started with pre-denaturation at 94^{o}C for 2 minutes followed by 35 cycles of denaturation step at 98^{o}C for 15 seconds, annealing step at 58^{o}C for 1 minute, and extension step at 68^{o}C for 1 minute. PCR products were electrophoresed in 1% agarose gel to check for the presence or absence of DNA bands. The purification and sequencing of PCR products were carried out by the company, FirstBase-Singapore.

Sequence alignment, variation analysis, and phylogenetic analysis

ChromasPro program (Technelysium Pty, Ltd) was used for contig assembly and chromatogram editing. The contigs were then compared with the DNA sequences in the gene bank using the BLAST (Basic Local Alignment Search Tool) to find similar DNA sequences. Alignments of the individual markers were assembled using the MEGA X program (Kumar *et al.* 2018). The MUSCLE alignment algorithm was applied (Edgar 2004). Manual observations on the aligned sequence were performed. The nucleotide gaps and differences found in the alignments were recorded as single variation characters. Phylogenetic reconstructions were conducted using the Maximum Likelihood (ML) method with Kimura-3-Parameter model,

pairwise deletion for gaps/missing data. Clade support values were obtained using bootstrap testing with 1000 replicates. The genetic distance for each marker was also calculated using pairwise distance in the MEGA X.

Table 1. Voucher information and GenBank numbers for all samples used

Species	Specimen voucher	Origin	Genbank accession numbers	
			trnL-F	ITS
Averrhoa dolichocarpa	NFSSs.n	Bogor Botanic Garden	MW559066	MW507601
Averrhoa dolichocarpa	EAW8831	Abepura, Papua	MW559062	-
Averrhoa leucopetala	RSSs.n	Bogor Botanic Garden	MZ043736	MW507599
Averrhoa dolichocarpa	SKS04	Bogor Botanic Garden	MW559061	MW507600
Averrhoa leucopetala	SKS05	Bogor Botanic Garden	MW559063	MW507598
Averrhoa leucopetala	ALT03	Bogor Botanic Garden	MZ043739	MZ014944
Averrhoa leucopetala	ALT01	Bogor Botanic Garden	MZ043740	MZ014945
Averrhoa dolichocarpa	ALBot	Gorontalo	MZ043737	MZ014943
Averrhoa dolichocarpa	ADBot	Abepura, Papua	MZ043738	MZ014942
Averrhoa carambola	NFSS1	Bogor Botanic Garden	MW559064	MW507602
Averrhoa carambola	ACR2	Java	MZ043744	-
Averrhoa carambola	ACR5	Java	MZ043743	MZ014948
Averrhoa carambola	ACXVI	Bogor Botanic Garden	MZ043742	MZ014947
*Averrhoa carambola**	Gravendeel 680	Java	JN620114.1	-
*Averrhoa carambola**		-	KX364202.1	-
*Averrhoa carambola**	AVCAR	-	EU437032.1	EU436863.1
*Averrhoa carambola**		India	-	KR905605.1
*Averrhoa carambola**		India	-	KR905606.1
*Averrhoa carambola**		India	-	KR905607.1
*Averrhoa carambola**		India	-	KR905608.1
*Averrhoa carambola**	J. Cai 13CS7239	China	KU569488.1	KU569491.1
*Averrhoa carambola**		India	-	MN511172.1
*Averrhoa carambola**	VanNeste811	America	-	MF348978.1
*Averrhoa carambola**		China	-	MG731074.1
*Averrhoa carambola**		China	-	MG731075.1
Averrhoa bilimbi	J.Wen 10211	Kolaka, SE Sulawesi	MW559065	-
Averrhoa bilimbi	AB3W	Java	MZ043741	MZ014946
*Averrhoa bilimbi**	AVBIL	-	AJ582291.1	EU436862.1
*Averrhoa bilimbi**		India	-	KR905596.1
*Averrhoa bilimbi**		India	-	KR905600.1
*Averrhoa bilimbi**		India	-	KR905595.1
*Averrhoa bilimbi**		India	-	KR905594.1
*Averrhoa bilimbi**		India	-	KR905597.1
*Averrhoa bilimbi**		India	-	KR905598.1
*Averrhoa bilimbi**		India	-	KR905599.1
*Averrhoa bilimbi**		India	-	KR905601.1
*Averrhoa bilimbi**		India	-	KR905602.1
*Sarcotheca monophylla**	DAPEN	-	EU437030.1	EU436860.1
*Dapania pentandra**	SARLAX	-	EU437031.1	EU436861.1

Notes: *: DNA sequences obtained from NCBI Genbank

RESULTS AND DISCUSSION

The ITS sequences of *Averrhoa* ranged from 693–713 bp including the 18S partial gene, the full sequence of ITS1, the full sequence of 5.8S gene and the full sequence of the ITS2 region. The *trnL-F* region consisted of *trnL* intron and *trnL-trnF* IGS ranging from 916–983 bp. Based on the pairwise distance analysis, ITS showed more variations than *trnL-F*. The overall mean distance of the ITS markers was 5%, while the overall mean distance of the *trnL-F* markers was 1%. This result indicated that the ITS evolution rate is faster than that of *trnL-F* in the genus *Averrhoa*.

In the ITS region, site-specific for the wild *Averrhoa* species were found at positions 76 (C), 148 (G), 369 (A), 425 (T), 435 (T), 498 (C), 562 (T), and 577 (T). The wild *Averrhoa* species showed differences in 15 sites. Specific mutation for *A. leucopetala* was found at positions 128 (-), 129 (-), 134 (C), 153 (A), 364 (C), 371 (C), 372 (C), 380 (T), 381 (G), 496 (G), 510 (C) and 511 (A) . Whereas, specific mutation for *A. dolichocarpa* was observed at positions 387 (T), 434 (C) and 457 (T) (Table 2). In the *trnL-F* region, there was only one specific mutation site for the wild *Averrhoa* species and four specific mutations for *A. dolichocarpa*, namely 319 (C), 561 (A), 709 (A), 746 (T) (Table 3).

Table 3. Nucleotide *specific* variation sites for *A. leucopetala* and *A. dolichocarpa* in *trnL-F* region

Taxon	Sequences				
	319	561	603	709	746
KX364202.1 *Averrhoa carambola*	A	C	G	G	C
KU569488.1 *Averrhoa carambola*	.	.	.	.	.
EU437032.1 *Averrhoa carambola*	.	.	.	.	.
NFSS1 *Averrhoa carambola*	.	.	.	.	.
ACR5 *Averrhoa carambola*	.	.	.	.	.
ACR2 *Averrhoa carambola*	.	.	.	.	.
JN620114.1 *Averrhoa carambola*	.	.	.	.	.
ACXVI *Averrhoa carambola*	.	.	.	.	.
J.Wen10211 *Averrhoa bilimbi*	.	.	.	.	.
AB3W *Averrhoa bilimbi*	.	.	.	.	.
AJ582291.1 *Averrhoa bilimbi*	.	.	.	.	.
ALBot *Averrhoa leucopetala*	.	.	A	.	.
ALT02 *Averrhoa leucopetala*	.	.	A	.	.
ALT01 *Averrhoa leucopetala*	.	.	A	.	.
RSSsn *Averrhoa leucopetala*	.	.	A	.	.
SKS05 *Averrhoa leucopetala*	.	.	A	.	.
NFSSsn *Averrhoa dolichocarpa*	C	A	A	A	T
ADBot *Averrhoa dolichocarpa*	C	A	A	A	T
EAW8831 *Averrhoa dolichocarpa*	C	A	A	A	T
SKS04 *Averrhoa dolichocarpa*	C	A	A	A	T

Based on those variations, it is possible to discriminate *A. leucopetala* and *A. dolichocarpa* using ITS and *trnL-F* sequences. There were more site variations in ITS than in *trnL-F*, and this is one of the characteristics of barcodes. The ITS marker could likely be used as a barcode for the genus *Averrhoa*. DNA barcode must have sequence variation, conserved flanking loci and short target DNA region (Stoeckle 2003). The recommended universal barcode region for plants is a combination of *mat*K and *rbc*L (CBOL 2009). For certain groups, barcode regions have a slow evolutionary rate and cannot be used to distinguish species. Some studies have instead focused on finding barcode loci to differentiate species of their specific interest groups (Kurian *et al.* 2020). The marker regions used in this study have previously been used on a close relative of *Averrhoa*, namely *Oxalis*. The barcode regions have provided sufficient variations to delimit the genus *Oxalis* (Oberlander *et al.* 2009; Vaio *et al.* 2016; Aoki *et al.* 2017).

The phylogenetic trees were reconstructed using maximum likelihood (ML), rooted by the outgroups, *S. monophyla* and *D. pentandra*. *Dapania*, *Sarcotheca,* and *Averrhoa* are closely related in the Oxalidaceae family (Veldkamp 1967), therefore we used *S. monophyla* and *D. pentandra* as outgroups in this study. The ML trees reconstructed based on the ITS and *trnL-F* regions resulted in the similar topology. The phylogenetic tree reconstructed based on the ITS region showed that *Averrhoa* was monophyletic, supported by 86% bootstrap. The *Averrhoa* genus consisted of two clusters (Fig. 1): cluster I consisted of *A. carambola* and *A. bilimbi*, and cluster II consisted of the four *Averrhoa* species.

A. carambola and *A. bilimbi* in cluster I were paraphyletic, grouped into the same cluster with strong bootstrap support (BS=96%). In cluster II, two subclusters separated wild *Averrhoa* species from cultivated *Averrhoa* species. *A. bilimbi* and *A. carambola* in this subcluster were also paraphyletic, grouped with strong bootstrap support (BS=100%). *A. dolichocarpa* and *A. leucopetala* were differentiated from cultivated *Averrhoa* species with 81% bootstrap support. *Averrhoa dolichocarpa* and *A. leucopetala* were distinguished from each other, supported by 99% bootstraps. These results supported the delimitation of *A. dolichocarpa* and *A. leucopetala* from cultivated *Averrhoa* species. However, the position of *A. carambola* and *A. bilimbi* on the tree was unclear. There were two groups of *A. carambola* and *A. bilimbi* but the species mixed up in each group.

Table 2. Nucleotide specific variation sites for *A. leucopetala* and *A. dolichocarpa* in ITS region

Taxon	Sequences																							
	76	128	129	134	148	153	364	369	371	372	380	381	387	425	434	435	457	460	496	498	510	511	562	577
KU569491.1 *Averrhoa carambola*	C	G	T	T	A	G	T	G	-	-	C	C	G	C	T	C	G	A	-	T	T	C	C	C
ACR5 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
ACXVI *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
EU436863.1 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	C	-	.	.	.	.	.
KR905605.1 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	C	-	.	.	.	.	.
KR905606.1 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	C	-	.	.	.	.	.
KR905607.1 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	C	-	.	.	.	.	.
KR905608.1 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	C	-	.	.	.	.	.
MF348978.1 *Averrhoa carambola*	-	-	-	-	-	-	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
MG731074.1 *Averrhoa carambola*	-	-	-	-	-	-	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
MG731075.1 *Averrhoa carambola*	-	-	-	-	-	-	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
NFSS1 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
MG731076.1 *Averrhoa carambola*	-	-	-	-	-	-	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
MN511172.1 *Averrhoa carambola*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
AB3W *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	C	-	.	.	.	.	.
EU436862.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905594.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905595.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	G	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905596.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905597.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905598.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905599.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905600.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905601.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
KR905602.1 *Averrhoa bilimbi*	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.
ALT03 *Averrhoa leucopetala*	T	-	-	C	G	A	C	A	C	C	T	G	.	T	.	T	.	C	G	C	C	A	T	T
SKS05 *Averrhoa leucopetala*	T	-	-	C	G	A	C	A	C	C	T	G	.	T	.	T	.	C	G	C	C	A	T	T
RSSsn *Averrhoa leucopetala*	T	-	-	C	G	A	C	A	C	C	T	G	.	T	.	T	.	C	G	C	C	A	T	T
ALT01 *Averrhoa leucopetala*	T	-	-	C	G	A	C	A	C	C	T	G	.	T	.	T	.	C	G	C	C	A	T	T
ALBot *Averrhoa leucopetala*	T	-	-	C	G	A	C	A	C	C	T	G	.	T	.	T	.	C	G	C	C	A	T	T
ADBot *Averrhoa dolichocarpa*	T	.	.	.	G	.	.	A	-	-	.	.	T	T	C	T	T	T	-	C	.	.	T	T
NFSSsn *Averrhoa dolichocarpa*	T	.	.	.	G	.	.	A	-	-	.	.	T	T	C	T	T	T	-	C	.	.	T	T
SKS04 *Averrhoa dolichocarpa*	T	.	.	.	G	.	.	A	-	-	.	.	T	T	C	T	T	T	-	C	.	.	T	T

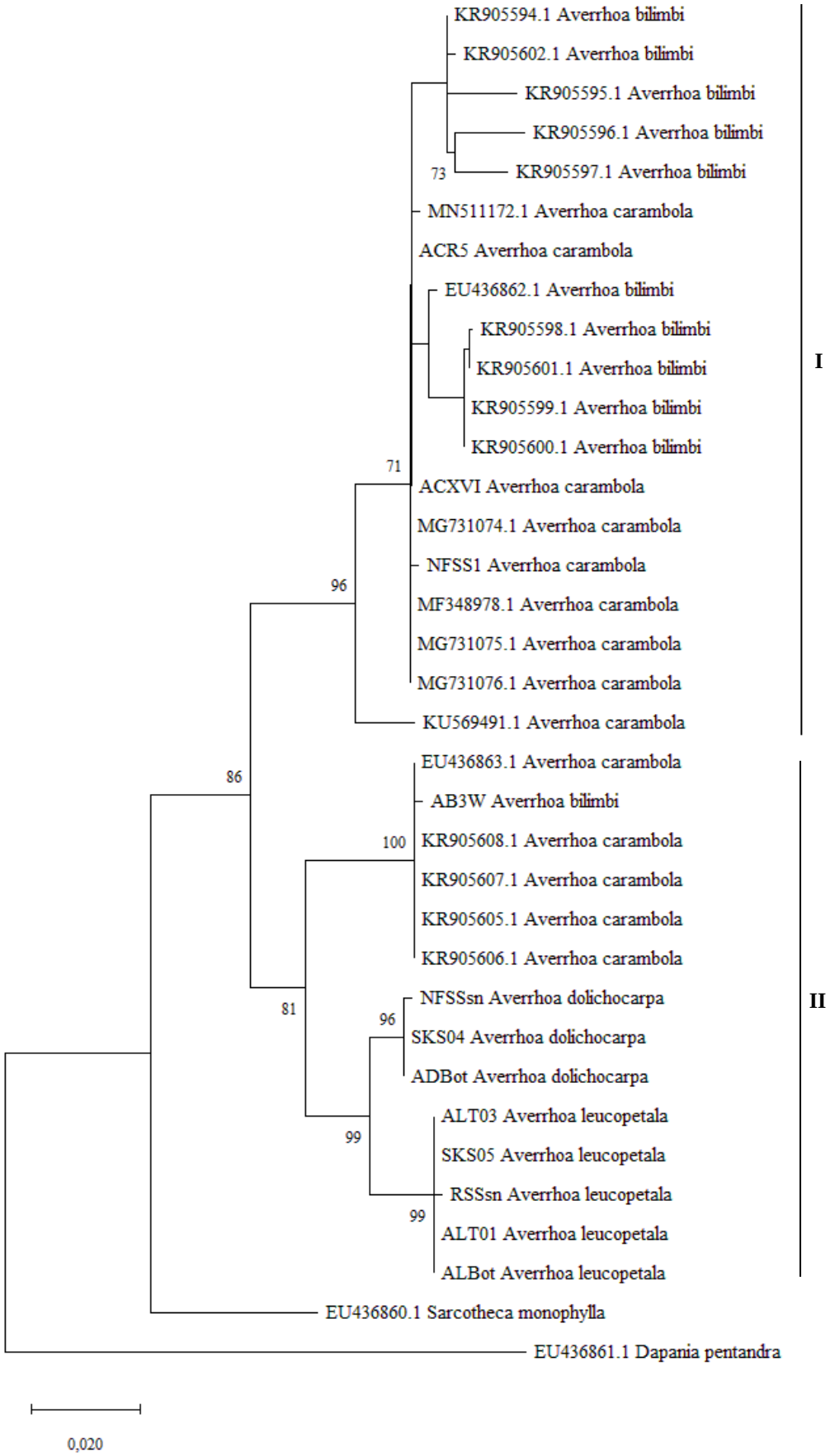

Figure 1. Phylogenetic tree of genus *Averrhoa* reconstructed from ITS data.
The bootstrap values for 1000 replicates are shown above or below the branches.

The phylogenetic tree reconstructed based on the *trnL-F* region revealed the similar topology (Fig 2). The genus comprised two clusters, with cluster I consisting of *A. carambola* and *A. bilimbi* supported by 52% bootstrap. Cluster II consisted of two sub-clusters where the cultivated *Averrhoa* species were separated from wild *Averrhoa* species. The sub-cluster consisting of *A. carambola* and *A. bilimbi* is supported by 52% bootstrap. Wild *Averrhoa* species congregated in another sub-cluster with strong bootstrap support (BS=73%).

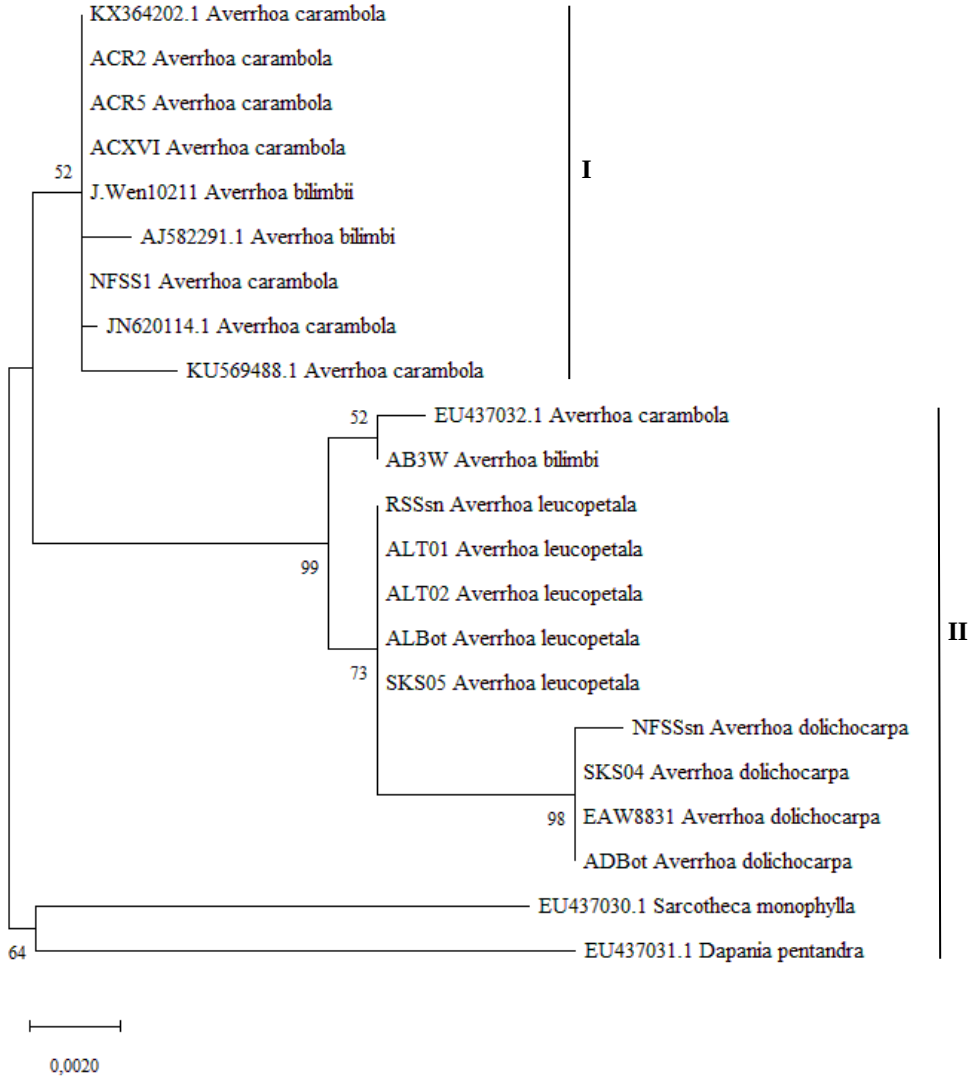

Figure 2. Phylogenetic tree of genus *Averrhoa* reconstructed from *trnL-F* data.
The bootstrap values for 1000 replicates are shown above or below the branches.

In the most comprehensive classification of Oxalidaceae, it is stated that *Averrhoa* is a distinct genus. Molecular evidence thus apparently corroborates the interpretation of *Averrhoa* as a distinct monophyletic group. The genus can be differentiated from its close relative genus by plurifoliolate/compound leaves (5 or more leaflets) and 3–7 ovules and seeds per locule (Veldkamp 1971; Cocucci 2004).

The two clusters in both trees (Figs. 1 & 2) were not congruent with morphological characters because *A. carambola* and *A. bilimbi* formed two paraphyletic groups. However, the morphological characters were congruent with the sub-clusters in cluster II. The characters that can be used to differentiate the two sub-clusters are inflorescence and sepal shape and size (Rugayah & Sunarti 2008). In the species description, it is stated that the flower is a specific character for each *Averrhoa* species. Based on the leaf characters, *A. dolichocarpa* is closer to *A. bilimbi,* but more similar to *A. carambola* based on its fruit characteristics. Whereas, *A. leucopetala* is more similar to *A. carambola* based on its leaf and fruit characters. However, when viewed from their generative properties (inflorescence, peduncle and rachis pedicels, sepal shape and size, and sepal surface), these wild starfruit species are closer to each other than to the cultivated *Averrhoa* species (Rugayah & Sunarti 2008).

The position of *A. leucopetala* and *A. dolichocarpa* on the phylogenetic tree was clear, separated from the cultivated *Averrhoa* species, and also distinguished from one another. This suggests that the delimitation of the

recently described *Averrhoa* species was supported by molecular evidence. The ITS and *trnL-F* regions can be used to delimit *A. dolichocarpa* and *A. leucopetala* from cultivated *Averrhoa* species and from one another. Morphologically, wild *Averrhoa* can be distinguished by the inflorescence clusters with crowded flowers on *A. dolichocarpa* and several flowers on *A. leucopetala*. Other characters that distinguish these two species are color of sepals, shape of petals, indumentum, and color of petals (Rugayah & Sunarti 2008).

The position of the cultivated *Averrhoa* species on the phylogenetic tree is puzzling. There are two paraphyletic groups at different levels, one at the cluster level and one at the sub-cluster. However, these two species are remarkably different in terms of the important morphological characters (Veldkamp 1971). Since *A. carambola* and *A. bilimbi* cannot be distinguished based on *trnL-F* and ITS regions, we suggest that further analysis using other molecular markers is required to carry out phylogenetic analysis in the genus *Averrhoa*.

CONCLUSIONS

Based on this study indicated that *Averrhoa* is a monophyletic group. Molecular evidence supported the delineation of *A. leucopetala* and *A. dolichocarpa* from their relative cultivated species. The *trnL-F* and ITS markers cannot be applied as a DNA barcode for the genus *Averrhoa*. However, these markers can be used to identify *A. dolichocarpa* and *A. leucopetala*.

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REFERENCES

Aoki S, Ohi-toma T, Li P, Fu C, Murata J. 2017. Phylogenetic, cytological, and morphological comparisons of *Oxalis* subsect. *Oxalis* (Oxalidaceae) in East Asia. Phytotaxa 324(3): 266–278. https://doi.org/10.11646/phytotaxa.324.3.3.

Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y. 2010. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. Plos One 5: e8613. https://doi.org/10.1371/journal.pone. 0008613.

Cocucci AA. 2004. Oxalidaceae. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants Volume VI. Springer-Verlag, Heidelberg, pp. 285–290.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792–1797.

Hutchinson J. 1959. The families of flowering plants, arranged according to a new system based on their probable phylogeny. 2 vols (2nd ed.). Macmillan. New York.

Kapsah, Dorly, Astuti IP. 2016. Morfologi dan viabilitas polen pada dua spesies belimbing hutan (*Averrhoa dolichocarpa* dan *A. leucopetala*). Buletin Kebun Raya 19(2): 79–90.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Molecular Biology and Evolution 35(6): 1547–1549. https://doi.org/ 10.1093/molbev/msy096.

Kurian A, Dev SA, Sreekumar VB, Muralidharan EM. 2020. The low copy nuclear region, RPB2 as a novel DNA barcode region for species identification in the rattan genus *Calamus* (Arecaceae). Physiology and Molecular Biology of Plants 26(9): 1875–1887. https://doi.org/10.1007/s12298-020-00864-5.

Mangunah, Qayim I, Astuti IP. 2013. Fenologi dan dinamika kandungan klorofil pada perbungaan dua jenis belimbing hutan (*Averrhoa dolicocarpa* dan *Averrhoa leucopetala*). Buletin Kebun Raya 16 (2): 101–112

Oberlander KC, Emshwiller E, Bellstedt DU, Dreyer LL. 2009. Molecular phylogenetics and evolution a model of bulb evolution in the eudicot genus Oxalis (Oxalidaceae). Molecular Phylogenetics and Evolution 51(1): 54–63. https://doi.org/10.1016/ j.ympev.2008.11.022.

Rugayah, Sunarti S. 2008. Two new wild species of *Averrhoa* (Oxalidaceae) from Indonesia. Reinwardtia 12(4): 325–331.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. PNAS 109: 6241–6246. https://doi.org/10.1073/pnas.1117018109.

Stoeckle M. 2003. Taxonomy, DNA, and the barcode of life. Bioscience 53(9): 796–797.

Sunarti S, Rugayah, Tihurua EF. 2008. Studi anatomi daun jenis-jenis *Averrhoa* di Indonesia untuk mempertegas status taksonominya. Berita Biologi 9(3): 253–257.

Taberlet P, Gielly L, Pautou G, Bouvet J. 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Molecular Biology 17(5): 1105–1109.

Vaio M, Gardner A, Speranza P, Emshwiller E, Guerra M. 2016. Phylogenetic and cytogenetic relationships among species of *Oxalis* section *Articulatae* (Oxalidaceae). Plant Systematics and Evolution 302: 1253-1265. https://doi.org/10.1007/s00606-016-1330-6.

Veldkamp JF. 1967. A revision of *Sarcotheca* Bl. and *Dapania* Korth. (Oxalidaceae). Blumea: Journal of Plant Taxonomy and Plant Geography 20: 519–543.

Veldkamp JF. 1971. Oxalidaceae, in: van Steenis CGGJ. (Ed.), Flora Malesiana Series I Vol 7. Noordhoff-Kolff, Jakarta, pp. 151–178.

White T, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky J, White T (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.

Yulita KS. 2011. Variasi dan kekerabatan genetik pada dua jenis baru belimbing (*Averrhoa leucopetala* Rugayah et Sunarti sp nov dan *A. dolichorpa* Rugayah et Sunarti sp nov., Oxalidaceae) berdasarkan profil Random Amplified Polymorphic DNA. Jurnal Biologi Indonesia 7(2): 321–330.